PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 26 April 1999 (26.04.99)	
International application No. PCT/US98/13591	**Applicant's or agent's file reference** F8061-8006
International filing date (day/month/year) 09 July 1998 (09.07.98)	**Priority date** (day/month/year) 11 July 1997 (11.07.97)
Applicant PANG, Peter, K., T. et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   11 February 1999 (11.02.99)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Sean Taylor
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

ATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

MURRAY, Robert, B.
Nikaido, Marmelstein, Murray & Oram LLP
Metropolitan Square
Suite 330
655 Fifteenth Street, N.W.
Washington, DC 20005-5701
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 26 April 1999 (26.04.99)	
Applicant's or agent's file reference F8061-8006	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US98/13591	09 July 1998 (09.07.98)	11 July 1997 (11.07.97)

Applicant
CV TECHNOLOGIES INC. et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,GB,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,EE,ES,FI,GE,GH,GM,HR,HU,ID,IS,KE,
   KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI,SL,TJ,TM,TR,TT,UA,
   UG,UZ,VN,YU,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

Sean Taylor

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: ROBERT B. MURRAY
NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
METROPOLITAN SQUARE
655 FIFTEENTH STREET, N.W. SUITE 330
WASHINGTON, DC 20005-5701

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of Mailing *(day/month/year)*	01 JUN 1999
Applicant's or agent's file reference: F8061-8006	REPLY DUE within TWO months from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/13591	09 JULY 1998	11 JULY 1997

International Patent Classification (IPC) or both national classification and IPC
IPC(6): C07K 1/00; A61K 35/32 and US Cl.: 530/840, 412; 424/548

Applicant
CV TECHNOLOGIES INC.

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	[X]	Basis of the opinion
II	[ ]	Priority
III	[X]	Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
IV	[X]	Lack of unity of invention
V	[X]	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	[ ]	Certain documents cited
VII	[ ]	Certain defects in the international application
VIII	[ ]	Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 11 NOVEMBER 1999

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MICHAEL BORIN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/408 (cover sheet) (January 1994)*

WRITTEN OPINION

International application No.
PCT/US98/13591

I. Basis of the opinion

1. This opinion has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".)*:

[X] the international application as originally filed.

[X] the description, pages 1-15 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.

[X] the claims, Nos. 1-15 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-8 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

3. [ ] This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:
NONE

WRITTEN OPINION

International application No.
PCT/US98/13591

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 5, 9-13

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 5, 9-13.

WRITTEN OPINION

International application No.
PCT/US98/13591

IV. Lack f unity f invention

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees the applicant has:

   ☐ restricted the claims. (See Supplemental Sheet)

   ☒ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1 not to invite the applicant to restrict or pay additional fees:

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

   ☐ all parts.

   ☒ the parts relating to claims Nos. 3,4,6,14.

WRITTEN OPINION

International application No.
PCT/US98/13591

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step r industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	3, 4	YES
	Claims	6,14	NO
Inventive Step (IS)	Claims	3,4	YES
	Claims	6, 14,	NO
Industrial Applicability (IA)	Claims	3,4,6, and 14	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 6,14 lack novelty under PCT Article 33(2) as being anticipated by US 5,618,925 or US 5,075,112 . '925 patent teaches use of shark cartilage extract as anti-angiogenic, tumor-regressing agent. See col. 2, bottom to col. 3, line 45. Also, '925 reviews prior art and describes that shark extract is known to inhibit cell prolidferation. Col. 2, lines 18-24. '112 patent teaches use of shark extract for inhibition of angiogenesis. See abstract. As angiogenesis or tumor include, as a cellular mechanism, increase in intracellular calcium, the referenced method read on the instant claim 6, drawn to method for treating a disease related to intracellular calcium. Further, as angiogenesis includes smooth muscle cell proliferation, the referenced method read on the instant claim 14, drawn to method for treating a disease related to vascular smooth muscle proliferation.

In regard to claim 15, the invention of the instant claim lacks an inventive step under PCT Article 33(3) as being obvious over '925 patent because it would be a matter of routine experimentation to select optrimal concentration ranges of components of cartilage composition.

In regard to extract of shark cartilage, it is anticipated by, or, in the alternative, is obvious over US 5,618,925, or US 4,473,55, or US 3,371,012. In particular, the '925 patent describes preparation of shark cartilage extract by extraction by water, and separation of unsolubilized material by centrifugation. Many other aqueous solutions can be used in lieu of water. See col. 4, last two paragraphs. The composition of the supernatant is disclosed in the table, column 5. Several different fractions of supernatant can be further separated, as disclosed in columns 10,11. Pharmaceutiical compositions comprising the shark cartilage extract were use for treatment of several disease conditions. See columns 13-18.

US 3,3710,12 teaches a shark cartilage extract prepared cartilage extraction with aqueous solution at 70super oC for 3 hours and filtration of the extract through a "Celite" column. See col. 3, lines 20-31.

US 4,473,551 teaches a shark cartilage extract prepared by extraction of shark cartilage with water at temperature 0-50oC for (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

IV. LACK OF UNITY OF INVENTION:

1. This response is made to a telephone Lack of Unity requirement (see telephone memorandum attached hereto or attached to a prior Written Opinion).

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
4-24 hours. The extraction procedure include repetitive extraction of the same portion of cartilage with new portions of water, to increase extraction of active components. See col. 2, lines 35-55.

Claims 3,4,6,14 meet the criteria for industrial applicability under PCT Article 33(4).

---------- NEW CITATIONS ----------
NONE

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference F8061-8006	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US98/13591	International filing date *(day/month/year)* 09 JULY 1998	Priority date *(day/month/year)* 11 JULY 1997
International Patent Classification (IPC) or national classification and IPC IPC(6): C07K 1/00; A61K 35/32 and US Cl.: 530/840, 412; 424/548		
Applicant CV TECHNOLOGIES INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☒ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11 FEBRUARY 1999	08 SEPTEMBER 1999
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL BORIN Telephone No. (703) 308-0196

JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATRIX

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/13591

I. Basis of the rep rt

1. This report has been drawn on the basis of (*Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments*):

[X] the international application as originally filed.

[X] the description, pages 1-15 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-15 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-8 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages none.

[X] the claims, Nos. none.

[X] the drawings, sheets/~~fig~~ none.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/13591

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 5, 9-13

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 5, 9-13.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/13591

IV. Lack of unity of inv ntion

1. In response to the invitati n to restrict or pay additi nal fees the applicant has:

- [ ] restricted the claims.
- [X] paid additional fees.
- [ ] paid additional fees under protest.
- [ ] neither restricted nor paid additional fees.

2. [ ] This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- [ ] complied with.
- [X] not complied with for the following reasons:

Please See Supplemental Sheet.

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- [ ] all parts.
- [X] the parts relating to claims Nos. 3,4,6,14.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/13591

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	3, 4, 14	YES
	Claims	6	NO
Inventive Step (IS)	Claims	3,4	YES
	Claims	6, 14	NO
Industrial Applicability (IA)	Claims	3,4,6, and 14	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claim 6 lacks novelty under PCT Article 33(2) as being anticipated by US 5,618,925 or US 5,075,112 . '925 patent teaches use of shark cartilage extract as anti-angiogenic, tumor-regressing agent. See col. 2, bottom to col. 3, line 45. Also, '925 reviews prior art and describes that shark extract is known to inhibit cell proliferation. Col. 2, lines 18-24. '112 patent teaches use of shark extract for inhibition of angiogenesis. See abstract. As angiogenesis or tumor include, as a cellular mechanism, increase in intracellular calcium, the referenced method read on the instant claim 6, drawn to method for treating a disease related to intracellular calcium.

Applicant points at particular processes occurring during angiogenesis, such as activation of tyrosine kinase and PKC, and asserts no intracellular messenger cascade occurs in these processes, and increase in intracellular calcium is not involved. First, activation of PKC is one of intracellular processes dependent on the rise in intracellular calcium. Second, angiogenesis involves a plurality of mechanisms beyond the several steps discussed by the applicant. Third, it is well known that any proliferation, angiogenesis included, involves increase in intracellular calcium. Further, the Written Opinion referred also to prior art drawn to treatment of tumor, which is also a disease related to intracellular calcium elevation. Finally, note that the claims are not drawn to affecting the intracellular calcium itself, but rather to the treatment of diseases related to intracellular calcium elevation, which any of known diseases is.

In regard to claim 14, the invention of the instant claim lacks an inventive step under PCT Article 33(3) as being obvious over '925 patent because it would be a matter of routine experimentation to select optimal concentration ranges of components of cartilage composition.

In regard to extract of shark cartilage, it is anticipated by, or, in the alternative, is obvious over US 5,618,925, or US 4,473,55, or US 3,371,012. In particular, the '925 patent describes preparation of shark cartilage extract by extraction by water, and separation of unsolubilized material by centrifugation. Many other (Continued on Supplemental Sheet.)

Supplem ntal Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

IV. LACK OF UNITY OF INVENTION:

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2, and 13.3 is not complied with for the following reasons:
This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1,2,7,8,15, drawn to shark cartilage extract.
Group II, claims 3, 4, drawn to first method of use, treating hypertension.
Group III, claim 6, drawn to third method of use, treating a disease related to intracellular Ca elevation.
Group IV, claim 14, drawn to fifth method of use, inhibiting smooth muscle proliferation.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I, II, IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The groups are related as product, method of use and method of making. Group I is the technical feature that links Groups I to III. Group I is not the contribution over the prior art because it is *prima facie* obvious over the references teaching shark cartilage extract, such as, for example, taught in US Patent 5,618,925. Therefore, the lack of unity is present because the linking technical feature is not a "special technical feature" as defined by PCT Rule 13.2.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
aqueous solutions can be used in lieu of water. See col. 4, last two paragraphs. The composition of the supernatant is disclosed in the table, column 5. Several different fractions of supernatant can be further separated, as disclosed in columns 10,11. Pharmaceutical compositions comprising the shark cartilage extract were use for treatment of several disease conditions. See columns 13-18.

US 3,3710,12 teaches a shark cartilage extract prepared cartilage extraction with aqueous solution at 70super oC for 3 hours and filtration of the extract through a "Celite" column. See col. 3, lines 20-31.

US 4,473,551 teaches a shark cartilage extract prepared by extraction of shark cartilage with water at temperature 0-50oC for 4-24 hours. The extraction procedure include repetitive extraction of the same portion of cartilage with new portions of water, to increase extraction of active components. See col. 2, lines 35-55.

Claims 3,4,6,14 meet the criteria set out in PCT article 33(4) for industrial applicability.

———— NEW CITATIONS ————

NONE

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 1/00, A61K 35/32

A1

(11) International Publication Number: **WO 99/02548**

(43) International Publication Date: 21 January 1999 (21.01.99)

(21) International Application Number: PCT/US98/13591

(22) International Filing Date: 9 July 1998 (09.07.98)

(30) Priority Data:
60/052,233 11 July 1997 (11.07.97) US

(71) Applicant *(for all designated States except US)*: CV TECHNOLOGIES INC. [CA/CA]; Campus Towers, Suite 308, 8625 – 112 Street, Edmonton, Alberta T6G 1K8 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PANG, Peter, K., T. [US/CA]; 205 5225 RR 232 Sherwood Park, Edmonton, Alberta T6B 1L5 (CA). SHAN, Jacqueline, J. [CA/CA]; 136 Twin Brooks Cove, Edmonton, Alberta T6J 6Y2 (CA). CHIU, Kam, W. [CA/CA]; Suite 1106, 11007 – 83 Avenue, Edmonton, Alberta T6G 0T9 (CA).

(74) Agents: MURRAY, Robert, B. et al.; Nikaido, Marmelstein, Murray & Oram LLP; Metropolitan Square, Suite 330, 655 Fifteenth Street, N.W., Washington, DC 20005–5701 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: A PREPARATION DERIVED FROM SHARK CARTILAGE FOR TREATMENT OF DISEASES RELATED TO EXCESSIVE PHF OR EXCESSIVE INTRACELLULAR CALCIUM

(57) Abstract

Shark cartilage extract has been shown to be an antagonist of parathyroid hypertensive factor (PHF). In view of this, shark cartilage extract can be used to treat conditions related to excessive PHF activity. Such diseases include hypertension and some other diseases related to intracellular calcium elevation. Methods for producing the shark cartilage extract and methods for administering the extract are disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

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A PREPARATION DERIVED FROM SHARK CARTILAGE FOR TREATMENT OF DISEASES RELATED TO EXCESSIVE PHF OR EXCESSIVE INTRACELLULAR CALCIUM

Field of the Invention

This invention relates to an anti-parathyroid hypertensive factor (anti-PHF) derived from shark cartilage. The compounds of the present invention can be used in the treatment of hypertension, and other diseases related to intracellular calcium elevation (e.g., non-insulin dependent diabetes mellitus; atherosclerosis; congestive heart failure; cancer (including breast, colon, kidney and leukemia); inflammatory bowel disease and asthma.

Background of the Invention

Hypertension is generally defined as the elevation of the systolic and/or diastolic arterial blood pressure above a nominal value of 140/90 mm Hg. Diseases associated with hypertension include artherosclerosis, hypertensive renal failure, stroke, congestive heart failure and myocardial infarction. Although numerous methods of treatment have been found to be effective in the reduction of arterial blood pressure, the etiology of essential hypertension remains essentially unknown. A genetic predisposition to hypertension is generally accepted, but the number of different drugs which have been found effective in the treatment of hypertension, and the fact that these drugs seem to operate by eliciting different pharmacological responses, suggests that there may be different primary causes for essential hypertension.

A number of studies have suggested that one or more circulating factors may play a role in the genesis or the maintenance of hypertension [See: Wright et al., A Hypertensive Substance Found in the Blood of Spontaneously Hypertensive Rats; *Life Sci.* 1984; 34:1521-1528; Dahl et al., Humoral transmission of Hypertension: Evidence from Parabiosis; *Circ. Res.* 1969; 24/25 (Supp. I): 21-23; Greenberg et al., Evidence for Circulating Factors as a Cause of Venous Hypertrophy in Spontaneously Hypertensive Rats; *Am. J. Physiol.* 1981; 241:H421-H430; Tobian et al., A Circulating Humoral Pressor Agent in Dahl S Rats with Salt Hypertension; *Clin. Sci.* 1979; 57:345s-347s; Zidek et al., Humoral Factors in the Pathogenesis of Primary Hypertension: *Klin. Wochenschr.* 1985; 63 (Supp.. II) D:94-96; Hirata et al.,

Hypertension Producing Factor in the Serum of Hypertensive Dahl Salt-Sensitive Rats; *Hypertension* 1984; 6:709-716]. For example, in parabiosis and cross-circulation experiments, an increase in blood pressure could be induced in normotensive animals by exposure to blood from hypertensive animals. The subcutaneous injection of erythrocyte-associated factor obtained from spontaneously hypertensive rates (SHR) has been shown to induce hypertension in normotensive Wistar-Kyoto (WKY) rats and an increase in blood pressure can be induced in normotensive, salt insensitive Dahl rats by injection of serum from hypertensive, salt-sensitive Dahl rats.

There have also been reports of circulating factors in both hypertensive rats and hypertensive humans which increase intracellular calcium [See: Banos et al., Two Factors Associated with Increased Uptake of Calcium in Platelets from Essential Hypertensive Patients; *Clin. Exp. Hypertens.* 1987; 9:1515-1530; Zidek et al., Effect of Plasma from Hypertensive Subjects on Ca Transport in Permeabilized Human Neutrophils; *Clin. Sci.* 1988; 74:53-56; Linder et al., Effects of a Circulating Factor in Patients with Essential Hypertension on Intracellular Free Calcium in Normal Platelets; *N. Eng. J. Med.* 1987; 316:509-513; Bruschi et al., Cytoplasmic Free Ca is Increased in the Platelets of Spontaneously Hypertensive Rats and Essential Hypertensive Patients; *Clin. Sci.* 1985; 68:179-184; Wright et al., Stimulation of Aortic Tissue Calcium Uptake by an Extract of Spontaneously Hypertensive Rat erythrocytes Possessing Hypertensive Properties; *Can. J. Physiol. Pharmacol.* 1986; 64:1515-1520]. Since vascular tone is influenced by the level of intracellular calcium, factors which increase blood pressure and factors which increase intracellular calcium may be related. There has been accumulating evidence suggesting the involvement of calcium regulating hormones in some forms of hypertension [See: L.M. Resnick, *Am. J. Med.* 82 (Supp. 1B), 16 (1987)]. Parathyroid hormone (PTH) is a calcium regulating hormone. Thirty percent or more of essential hypertensive patients fall into a subgroup characterized by increased levels of immunoreactive parathyroid hormone (ir-PTH). [See: Laragh et al., *Kidney Int.* 34, (Supp. 35), S162 (1988)]. An increase in PTH levels has been reported in SHR rats [See:

McCarron et al., *Hypertension* 3 (Supp. 1), I162 (1981)] and it has been observed that hyperparathyroid patients often exhibit hypertension, the severity of which can, in most cases, be reduced by parathyroidectomy [See: Hellstrom et al., *Brit. J. Urol.* 30, 13 (1958)]. Similar results from parathyroidectomy have also been reported in SHR rats. [See: Schleiffer et al., *Jap. Circ. J.* 45, 1272 (1981)]. Various investigators have suggested that PTH contributes to the development of essential hypertension, although exogenous administration of PTH causes a reduction in blood pressure in mammals and other vertebrates [See: Pang et al., *Gen. Comp. Endocrinol.* 41, 135 (1980)]. The vasodilating action of PTH is also related to a specific region of the molecule separate from the region mediating hypercalcemic effects [See: Pang et al., *Endocrinology*, 112, 284 (1983)]. PTH has also been shown to inhibit calcium entry into vascular smooth muscle [See: Pang et al., *Life Sci.*, 42, 1395 (1988)] through L-type calcium channels [Wang et al. FEBS, Vol. 282, No. 2, pp. 331-334 (1991)]. This paradox is further heightened by the fact that hypertensive patients with increased PTH levels exhibit decreased serum ionized calcium levels [See: Resnick et al., *New Engl. J. Med.*, 309, 888 (1983); Hvarfner et al., *Acta Med. Scand.* 219, 461 (1986)]. It would be expected that the serum ionized calcium levels would be elevated if PTH were primarily elevated.

The existence of a circulating factor in the blood of the SHR rat was confirmed by the studies reported in *Am. J. Hypertens.*, 2, 26-31 (1989). In these studies, an increase in the blood pressure of WKY and SD rats when plasma from SHR rats was injected into the normotensive rats either by infusion or by bolus injection was shown. In addition, it has been shown that the uptake of ^{45}Ca by sections of the tail artery of a rat, *in vitro*, increased in a dose-dependent manner as the concentration of SHR plasma was increased in a buffer-based medium. The results of these experiments clearly show that an increase in blood pressure and an increase in calcium uptake in the cells were both dose-dependent on the amount of SHR plasma present and available in the system. Curiously, the onset of both events was delayed, and gradual, whereas known endogenous pressor agents such as

norepinephrine, angiotensin II and vasopressin have been observed to increase blood pressure quite rapidly after administration. The known endogenous pressor agents exhibit about a 1-2 minute onset in the increase of blood pressure and increase in calcium uptake in the cells whereas parathyroid hypertensive factor has a 20-30 minute delay before such onset. Another result observed in these studies was that when the infusion of SHR plasma was stopped and substituted with plasma from normotensive rats, the observed blood pressure decreased quite rapidly to the baseline. The decrease observed precluded a simple volume effect. In a related experiment, dialyzed plasma from hypertensive human subjects was infused into normotensive SD rats and shown to produce hypertension. Plasma from these subjects also increased calcium uptake in rat tail arteries *in vitro*. Dialyzed plasma from normotensive patients produced no significant increase in blood pressure.

The origin of the circulating factor was unknown, but the anecdotal reports that PTH was elevated in hypertensive rats suggested the parathyroid gland as a target of investigation. Parathyroidectomies of SHR rats were found to reduce blood pressure and plasma from the SHR rats which had been parathyroidectomized did not cause elevation of blood pressure in normotensive rats. Conversely, transplantation of parathyroid glands from SHR rats to normotensive Sprague-Dawley (SD) rats resulted in an increase in blood pressure and the appearance of the factor in the plasma, as shown by infusion of the isolated plasma into other normotensive rats. [Pang and Lewanczuk, *Amer. J. Hypertens.* 2, 898 (1989)].

On the basis of these studies, the parathyroid was determined to be the origin of the circulating factor and the name "Parathyroid Hypertensive Factor" or PHF was proposed for the substance which causes an elevation in blood pressure.

The isolation and purification of a circulating factor, having its origin in the parathyroid gland, has been demonstrated in SHR rats and in many humans having essential hypertension and is the subject matter of related patent application Serial No. 603,745 filed November 21, 1990, which is a

continuation-in-part of patent application Serial No. 327,450, filed March 22, 1989, now abandoned. The disclosure of the related patent applications are incorporated herein by reference for their teachings, including the teachings of purification of parathyroid hypertensive factor.

As described in the aforementioned related patent applications, PHF has been shown to regulate extracellular calcium uptake, and can be inhibited by increases in dietary calcium levels. PHF has been isolated and a method for screening for PHF using antibodies raised against PHF have been described. PHF has a molecular weight of approximately 2,700 daltons and has the property of delayed onset of an increase in blood pressure of a normotensive rat when administered thereto, the increase in blood pressure temporally correlating with an increase in extracellular calcium uptake by vascular smooth muscle. From bioassay data, the factor in humans and rats has been found to be substantially similar.

Vascular hypertrophy has been implicated in the pathophysiology of a number of cardiovascular diseases including essential hypertension. Vascular smooth muscle proliferation could account for vascular hypertrophy and increased vascular tone. It was reported that PHF increased vascular smooth muscle cell proliferation through a mechanism independent of intracellular calcium regulation (Shan et al., Abstract in 17th Scientific Meetings of the International Society of Hypertension, Amsterdam, 7-11 June 1998).

Antagonists of PHF have been found by the present inventors. The present inventors have unexpectedly found that shark cartilage, known in the art to contain a substance which inhibits tumor angiogenesis [Lee et al., Science, vol. 221, pp.1185-1187, (1983)] and to contain an anti-inflammatory component [Schinitsky U.S. Patent No. 4,473,551], acts as an antagonist of PHF resulting in a decrease in blood pressure and affecting intracellular calcium regulation. The present inventors have also found that shark cartilage extract inhibited VSMC proliferation in SHR rats or in WKY rats induced by PHF. In view of this, shark cartilage extract according to the present invention is expected to be useful for treating hypertension and other diseases related to intracellular calcium elevation.

Detailed Description of the Invention

The present inventors have found that an extract prepared from shark cartilage produces a decrease in blood pressure. The shark cartilage extract is believed to contain a parathyroid hypertensive factor antagonist which binds to the parathyroid hypertensive factor site without activating parathyroid hypertensive factor activity.

The shark cartilage extract can be obtained by further purifying commercially available shark cartilage which has been cleaned, dried and milled to a fine powder. The dried ground shark cartilage is first extracted with H_2O at a temperature between 4-120°C (preferably 95°C) for 2-4 hours (preferably 2 hours). The ratio of solute to solvent is between 1:8 and 1:12. The resulting suspension is then cooled to between 40-60°C (preferably 50°C) and centrifuged at about 5200 to 5700 rpm to separate the suspension into a supernatant (#1) and pellet. The supernatant (#1), which contains about 8% solids, is held in a cooling tank at 4-8°C while the pellet is subjected to a second extraction. In the second extraction the pellet is extracted with H_2O at a temperature between 4-120°C (preferably 95°C) for 2-4 hours (preferably 2 hours). The ratio of solute to solvent is 1:4 - 1:6 (based on starting material). The resulting suspension is then cooled to between 40-60°C (preferably 50°C) and centrifuged at about 5200 to 5700 rpm to separate the suspension into a supernatant and pellet. The supernatant is then pooled with the supernatant from the first extraction and spray dried to obtain the purified shark cartilage extract of the present invention.

The extract of the present invention may be administered to a warm blooded mammal in need of such treatment, by parenteral, topical, oral or rectal administration or by inhalation. The extract may be formulated for parenteral or oral dosage by compounding the extract with a conventional vehicle, excipient, binder, preservative, stabilizer, color, agent or the like as called for by accepted pharmaceutical practice.

For parenteral administration, a 1-10 ml intravenous, intramuscular or subcutaneous injection would be given one to four times daily. The injection would contain the shark cartilage extract of the present invention in an

aqueous isotonic sterile solution or suspension optionally with a preservative such as phenol or a solubilizing agent such a ethylenediaminetetraacetic acid (EDTA). Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. Synthetic monoglycerides, diglycerides, fatty acids (such as oleic acid) find use as fixed oils in the preparation of injectables.

For rectal administration, the extract can be prepared in the form of suppositories by mixing with a suitable non-irritating excipient such as cocoa butter or polyethylene glycols.

For topical use, the extract can be prepared in the form of ointments, jellies, solutions, suspensions or dermal adhesive patches.

In a powdered aerosol, the extract may be administered by a Spinhaler turbo-inhaler device obtained from Fisons Corporation of Bedford, Massachusetts, at a rate of about 0.1 to 50 mg per capsule, 1 to 8 capsules being administered daily for the average human. In a liquid aerosol, the extract is administered at the rate of about 100 to 1000 micrograms per "puff" or activated release of a standard volume of propellant. The liquid aerosol would be given at the rate of 1 to 8 "puffs" per day with variation in dosages due to the severity of the conditions being treated, the weight of the patient and the particle size distribution of the aerosol. A fluorinated hydrocarbon or isobutane can be used as propellants for liquid aerosols.

Daily doses are in the range of about 0.01 to about 200 mg per kg of body weight (preferably 1-10 mg/kg body weight) depending on the activity of the specific compound, the age, weight, sex and conditions of the subject to be treated, the type and severity of the disease, the frequency and route of administration. As would be well known, the amount of active ingredient that may be combined with the carrier materials to produce a single dosage will vary depending upon the host treated and the particular mode of administration.

The shark cartilage extract can also be combined with drugs known to be effective for treating the condition in question. For example, to treat

hypertension, shark cartilage extract can be combined with known antihypertensive drugs such as calcium channel blockers (e.g. verapamil, nifedipine and diltiazem).

In addition to the treatment of essential hypertension, the extract of the present invention can be used to treat other diseases which may include but do not necessarily include hypertension as a primary symptom. For example, noninsulin dependent diabetics are frequently hypertensive. Conversely, hypertensives frequently show an impaired glucose tolerance. Thus, shark cartilage extract is expected to be useful for treating hypertension and other diseases related to intracellular calcium elevation.

The present invention is intended to encompass the isolation, identification and synthetic production of the active ingredient from shark cartilage extract.

The following examples illustrate but are not intended to limit the present invention. Various modifications may be apparent to those skilled in the art without deviating from the scope of this invention.

Example 1

Extraction of Shark Cartilage

Cleaned, dried, ground shark cartilage was purchased. The dried ground shark cartilage was first extracted with H_2O at a temperature between 85° to 90°C for 2 hours. The ratio of solute to solvent was 1:8. The resulting suspension was then cooled to 50°C and centrifuged at 5200 rpm (3245 g) to separate the suspension into a supernatant and pellet. The supernatant, which contained about 8% solids, was held in a cooling tank at 4°C while the pellet was subjected to a second extraction. In the second extraction the pellet was extracted with H_2O at 95°C for 3 hours. The ratio of solute to solvent was 1:4.8 based on the starting material. The resulting suspension was then cooled to 50°C and centrifuged at 5200 rpm (3245 xg) to separate the suspension into a supernatant and pellet. The supernatant, which contained about 3% solids, was pooled with the supernatant from the first extraction and spray dried to obtain the purified shark cartilage extract of the present invention.

Example 2

Effect of bolus injection of shark cartilage extract (1mg/kg) in SHR and SD rats

Six (6) spontaneously hypertensive rats (SHR) and three (3) Sprague-Dawley (SD) rats were given an intravenous bolus injection of shark cartilage extract denoted as DFI-40. Five (5) spontaneously hypertensive rats (SHR) and three (3) Sprague-Dawley (SD) rats were given an intravenous bolus injection of shark cartilage extract denoted as DF II-40. The shark cartilage extract was administered at a dosage of 40 mg/kg body weight. Blood pressure was measured for 90 minutes after the injection. As shown in Figure 1, the shark cartilage extract produced no effect in SD rats but decreased the blood pressure in SHR rats.

Example 3

Effect of gavage administration of shark cartilage extract on SHR and SD rats

Three groups of SHR rats were gavage fed with three different doses of shark cartilage extract (10, 20 and 40 mg/kg) from batch DF II-53. 11 rats were administered 10 mg/kg body weight shark cartilage extract, 4 rats were administered 20 mg/kg body weight shark cartilage extract and 4 rats were administered 40 mg/kg body weight shark cartilage extract. Blood pressure was measured for 90 minutes after administration. As shown in figures 2, 2a and 2b, all of the rats showed a decrease in blood pressure which was dose related. In rats given higher doses, (20-40 mg/kg body weight), the rate of decrease in blood pressure is greater with the maximum decrease being reached at around 50-60 minutes (figure 2a). After 50-60 minutes, the blood pressure fluctuates possibly due to the blood pressure regulating mechanisms of the rat.

Example 4

Effect of PHF on the blood pressure of SD rats in the presence and absence of shark cartilage extract

Seven (7) SD rats were administered 1 ml equivalent of PHF by IV bolus injection. Six (6) SD rats were administered 1 ml equivalent of PHF by IV bolus injection and 10 minutes later 40 mg/kg body weight of shark cartilage extract (DF II-53) was administered. Blood pressure was measured for 90 minutes following the injections. As shown in figure 3, PHF produces a delayed increase in blood pressure and the shark cartilage extract counteracts this response.

Example 5

Effect of PHF on vascular smooth muscle cell (VSMC) proliferation in the presence and absence of shark cartilage extract

The tail artery of male West Kyoto (WKY) rats or Spontaneous Hypertensive Rats (SHR) (100-200 g body weight) was dissected out and immersed in the cold Ca-omitted and Mg-omitted Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY). The tail artery was digested twice with HBSS enzyme solution II and I consecutively. Each digestion lasted for 1 hour. HBSS enzyme solution I contained CaCl2 (0.2 mM), collagenase/dispase (1.5 mg/ml) (Boehringer Mannheim GmbH, West Germany), elastase (Type I, 0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO), trypsin inhibitor (Type I, 1 mg/ml) (Sigma Chemical Co.) and bovine serum albumin (BSA) (fatty acid free, 2 mg/ml) (Sigma Chemical Co.). HBSS enzyme solution II contained collagenase (Type II, 1 mg/ml) (Sigma Chemical Co.), trypsin inhibitor (0.3 mg/ml) and BSA (2 mg/ml). The cell suspension were then seeded into 96 flat-bottom well tissue culture plates in DMEM medium with 10% FCS and incubated at 37°C in a humidified atmosphere with 5% CO2 in air for 36 hours to allow cells attachment to the bottom of the plate. The medium was changed to DMEM with 0.4% of FCS to render the cells quiescent for 2-4 days. This procedure synchronised cells in the Go-G1 boundary. PHF and shark cartilage were dissolved in DMEM with 10% FCS. PHF alone or PHF plus shark cartilage was added into the quiescent cells. After incubation for 36 hours, the cells were pulsed with 3H-thymidine (0.2 (/well and incubated for another 24 hours. The medium was then removed

and the cells were washed twice with HBSS followed by a 15-30 minutes incubation with 0.1% of trypsin at room temperature. The cells were then harvested onto filter paper by the cell harvest. The amount of radioactivity incorporated into cells was determined using a liquid scintillation counter. As shown in figure 4, PHF stimulated VSMC cell proliferation in WKY rats. Figure 5 shows that the stimulating effect of PHF on VSMC in WKY rats can be inhibited by shark cartilage extract. Figure 6 shows that shark cartilage inhibited VSMC proliferation of SHR rats.

Example 6

Chemical composition of shark cartilage extract

(1). Determination of Protein Content

Total protein content is determined using the BCA method. The BCA Protein Assay Reagent is purchased from the PIRRCE. A standard curve of protein standards of known concentration can be constructed by using the BSA (bovine serum albumin) standard solution provided with the BCA Protein Assay Reagent Kit. Twenty-four glass tubes were set in three rows and seven columns for standard samples and another four tubes were set for spectrophotometer calibration. Ninety-five, 90, 80, 70, 60, 50, 40, and 30μl of 0.9% sodium chloride was applied into the first row of the tubes respectively. The same procedure was repeated for the second and third rows. Five, 10, 20, 30, 40, 50, 60, and 70μl of standard protein (provided with the kit and at a concentration of 2mg/ml) were applied into the first row of tubes containing 0.9% sodium chloride. The same procedure was repeated for the second and third rows. Two mls of the Working Reagent, which is a mixture of 50 parts of Reagent A and 1 part of Reagent B was then added to each tube. All samples were well mixed and incubated at 37°C for 30 min. Protein was determined by measuring the absorbency at 562 nm with a spectrophotometer (Model PU 8620 UV/VIS/NIR, Philips). The mean values of each concentration of standards were calculated and a standard curve was constructed by using the Analysis of the Regression Line No. 5, Pharmcologic

Calculation System-Version 4.2A. This standard curve was used to determine the protein concentration for each unknown sample.

1% of shark cartilage extract solution was prepared in double distilled (DD) water. The protein concentration (mg/ml) of the sample solution was calculated by using the standard curve and shark cartilage protein content by percentage was calculated by using the following formulation:

Protein %(w/w)=sample protein concentration (mg/ml) x dilution factor (2.5)/sample concentration (10mg/ml) x 100.

To obtain accurate data for the standard curve and shark cartilage sample, the procedure for standard curve construction and shark cartilage extract protein content determination were carried out simultaneously, the Working Reagent was the last reagent added into all tubes for the standard protein samples and the shark cartilage sample.

The protein content is 15.11(2.79 (%) in a total of 16 batches of shark cartilage extract.

(2). Determination of mucopolysacchrides

The method was adapted from P.Whiteman (Biochem. J. 131:351-357, 1973) and E. Gold (Analytical Biochemistry 99: 183-188, 1979). Standard sample Chondroitin Sulfate C was purchased from Sigma chemical Co., Cat No.C-4384, Lot No. 21H0103. Standard or samples were prepared by dissolving 10mg Chondroitin Sulfate C or shark cartilage extract in 50ml DD water. Reaction reagent was prepared by dissolving 20mg Aleian Blue 8GX in 20ml buffer (5.07g magnesium chloride and 3.4 g sodium acetate in 500 ml water) and 0.2ml acetic acid. A series of shark cartilage extract samples ranging 40-200μg in 1 ml was added into a 50ml-plastic tube respectively. One ml of reaction reagent was added into these tubes. The mixture was equilibrated for 2 hours at room temperature with stirring. Twenty ml of 95% ethanol was added followed by centrifugation. After decanting the supernatant, three ml of 0.2M calcium chloride was added to the precipitate. The mucopolysaccharide content was determined by measuring the absorbency of the calcium chloride solution of precipitate at 620 nm.

The mucopolysaccharide content was 50.33(2.25(%) in 6 batches of shark cartilage extract.

(3). Isolation and determination of chondroitin C

The method was adapted from L. Roden, et al., Methods in Enzymology (1972), Vol. 28, Complex Carbohydrates part B, ed. by V. Ginsburg. Amberlite IR-120 Plus was purchased from Sigma Chemical Co., Cat No. IR-120 Plus. Calcium acetate buffer was prepared by adding 1.2L DD water to 62.5g calcium acetate. pH was adjusted to 4.5 with 35.5ml glacial acetic acid. Two grams of shark cartilage extract was added to 400ml of calcium acetate buffer in a 2L-glass flask. Sample solution was heated in a water bath at 37°C for 20 min, then cooled to room temperature. Ethanol (100%,116.25ml) was added to the sample solution very slowly with vigorous stirring at room temperature. Set the flask at 4°C bath for 3 hours followed by centrifugation (11,000 rpm, 19,000g) for 15 min at 4°C. The precipitate was dissolved in DD water and freeze dried. The supernatant was warmed to room temperature and was added into 80ml of ethanol (100%) slowly with vigorous stirring. The flask was set in 4°C bath again overnight with slow stirring. The solution was centrifuged at 4°C (11,000 rpm) for 15 min. The second precipitate was dissolved in DD water and freeze dried, The supernatant was warmed to room temperature and 100ml ethanol was added slowly with vigorous stirring. Again the flask was set in 4°C bath overnight with slow stirring followed by centrifugation at 11,000 rpm for 15 min. DD water (125 ml) was added to the third precipitate which was applied to an Amberlite IR-120+(Na+ form) column (2.5 x 16cm, about 60g of Amberlite IR-120 Plus). The column was washed with 75ml of DD water. After adding 1.168g NaCl to make the solution 0.1M in salt 3 volumes (600ml) of absolute ethanol was applied with vigorous stirring. Again, the flask was placed in 4-°C bath overnight followed by centrifugation (11,000 rpm) at 4° C for 15 min. The last precipitate was dissolved in DD water and freezes dried. The weight of last precipitate represents the amount of chondroitin sulfate C.

The chondroitin sulfate C content was 5.9(1.98(%) in 2 batches of shark cartilage extract.

Brief Description of the Drawings

Figure 1 shows the results of an IV bolus injection of shark cartilage extract in SHR and SD rats. As shown in Figure 1, the shark cartilage extract produced no effect in SD rats but decreased the blood pressure in SHR rats.

Figure 2 shows the results of gavage administration of shark cartilage extract in SHR rats. As shown in figure 2, the shark cartilage extract produced a decrease in blood pressure in all of the rats.

Figures 2a and 2b show that the decrease in blood pressure is dose related and the maximum decrease is reached at around 50-60 minutes.

Figure 3 shows that PHF produces a delayed increase in blood pressure and the shark cartilage extract counteracts this response.

Figure 4 demonstrates that PHF stimulated VSMC of WKY rats proliferation in a dose-dependent manner. At the doses of 0.625x10-3, 1.25x10-3 and 2.5x10-3 absorption unit, PHF increased cell proliferation by 120(8.5 (%) ($P<0.05$, n=16), 137.91(12(%)($P<0.01$, n=16) and 181.9(14.3 (%) ($P<0.05$, n=16) respectively.

Figure 5 shows the effect of PHF on VSMC of WKY rats proliferation in the presence of shark cartilage extract. At dose of 50 (g/ml, shark cartilage extract significantly inhibits VSMC proliferation induced by PHF.

Figure 6 shows the effects of shark cartilage extract on VSMC of SHR rats. At the doses of 5, 50 and 500 (g/ml, shark cartilage extract inhibits VSMC proliferation in a dose-dependent manner.

CLAIMS

1. A shark cartilage extract with anti-parathyroid hypertensive factor (PHF) activity.

2. The shark cartilage extract with anti-PHF activity according to claim 1, wherein the shark cartilage extract is produced by the following steps:

extracting cleaned, dried, ground shark cartilage with H_2O at a temperature between 4-120°C for 2-4 hours,

cooling the resulting suspension to between 40-60°C,

centrifuging the cooled suspension at between 5200 to 5700 rmp to separate the suspension into supernatant 1 and pellet,

holding the supernant 1 in a cooling tank at 4-8°C,

extracting the pellet a second time with H_2O at a temperature between 4-120°C for 2-4 hours,

cooling the resulting suspension to between 40-60°C,

centrifuging the cooled suspension at between 5200 to 5700 rpm to separate the suspension into supernatant 2 and pellet,

pooling supernatant 1 with supernatant 2, and

spray drying the pooled supernatants to obtain the shark cartilage extract.

3. A method for treating hypertension comprising administering to a patient in need of such treatment, an anti-hypertensive effective amount of shark cartilage extract.

4. The method according to claim 3, wherein said amount is 0.1-20 mg/kg body weight.

5. A method for treating a disease related to excessive PHF comprising administering to a patient in need of such treatment, an amount of shark cartilage extract effective to treat said disease.

6. A method for treating a disease related to intracellular calcium elevation comprising administering to a patient in need of such treatment, an amount of shark cartilage extract effective to treat said disease.

7. A pharmaceutical composition comprising shark cartilage extract

with anti-parathyroid hypertensive factor activity and a pharmaceutically acceptable carrier.

8. A pharmaceutical composition comprising shark cartilage extract with anti-parathyroid hypertensive factor activity, an antihypertensive substance and a pharmaceutically effective carrier.

9. A method for counteracting the activity of parathyroid hypertensive factor, comprising administering an effective amount of shark cartilage extract with anti-parathyroid hypertensive factor activity.

10. A method for producing a purified shark cartilage extract with anti-parathyroid hypertensive factor activity, comprising the steps of:

extracting cleaned, dried, ground shark cartilage with H_2O at a temperature between 4-120°C for 2-4 hours,

cooling the resulting suspension to between 40-60°C,

centrifuging the cooled suspension at between 5200-5700 rpm to separate the suspension into supernatant 1 and pellet,

holding the supernatant 1 in a cooling tank at 4-8°C,

extracting the pellet a second time with H_2O at a temperature between 4-120°C for 2-4 hours,

cooling the resulting suspension to between 40-60°C,

centrifuging the cooled suspension at between 5200 to 5700 rpm to separate the suspension into supernatant 2 and pellet,

pooling supernatant 1 with supernatant 2, and

spray drying the pooled supernatants to obtain the shark cartilage extract.

11. The method according to claim 10, wherein said extracting steps are conducted at 95°C for 2 hours.

12. The method according to claim 10, wherein a decanter centrifuge is used in said centrifuging steps.

13. The method according to claim 10, further comprising concentrating the pooled supernatants until a solids content of between 8 - 10% is reached.

14. A method for inhibiting vascular smooth muscle cell proliferation, comprising administering to a patient in need of such treatment, an amount of the composition according to claim 7 effective to inhibit vascular smooth muscle cell proliferation.

15. The extract according to claim 2, wherein said extract is composed of 5-30% protein, 15-80% mucopolysaccharides and 1-20% Chondroitin Sulfate C.

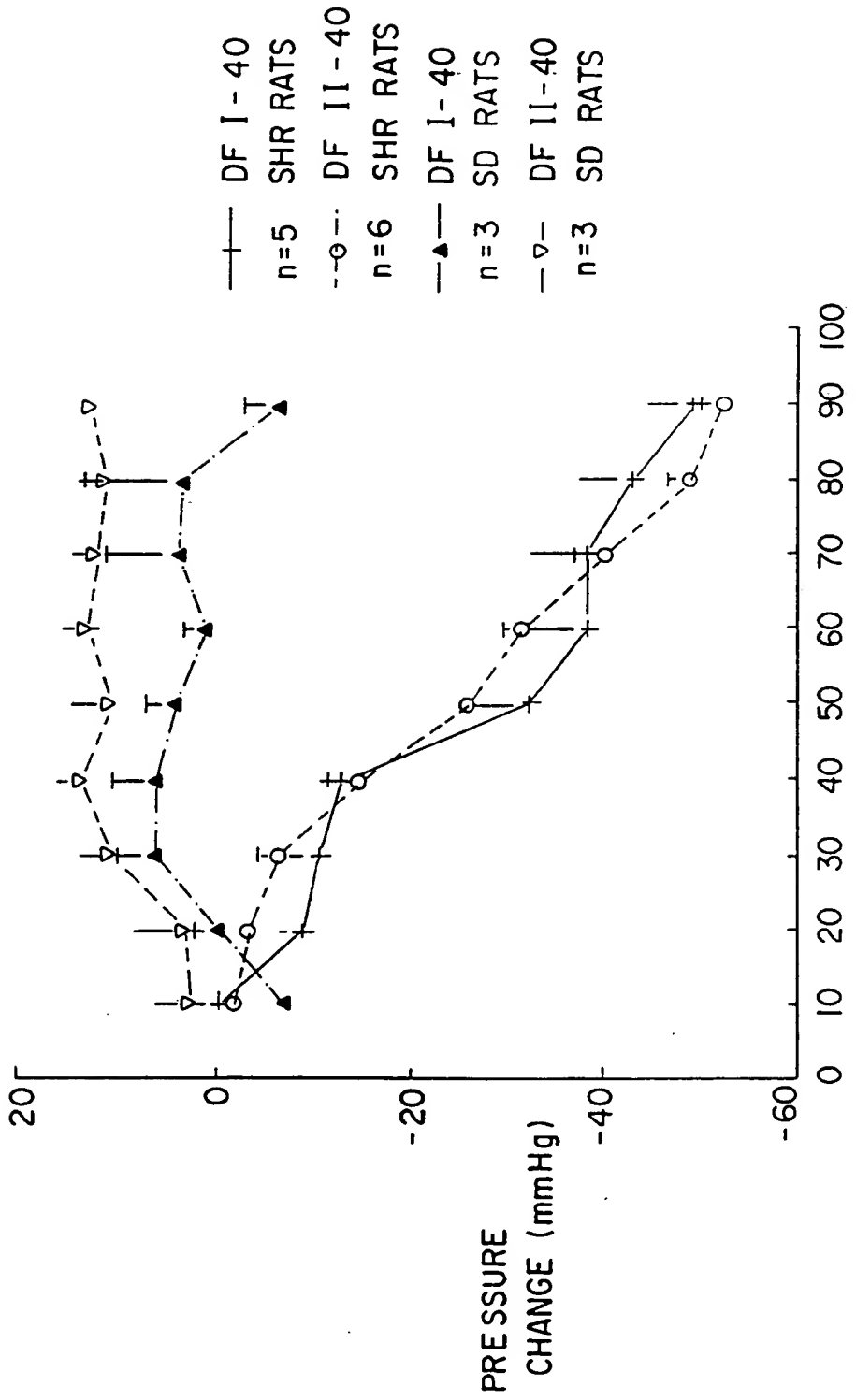
DF I-40
n=5 SHR RATS
DF II-40
n=6 SHR RATS
DF I-40
n=3 SD RATS
DF II-40
n=3 SD RATS
PRESSURE CHANGE (mmHg)
20
0
-20
-40
-60
0 10 20 30 40 50 60 70 80 90 100
TIME AFTER INJECTION (mins)

Fig. 1

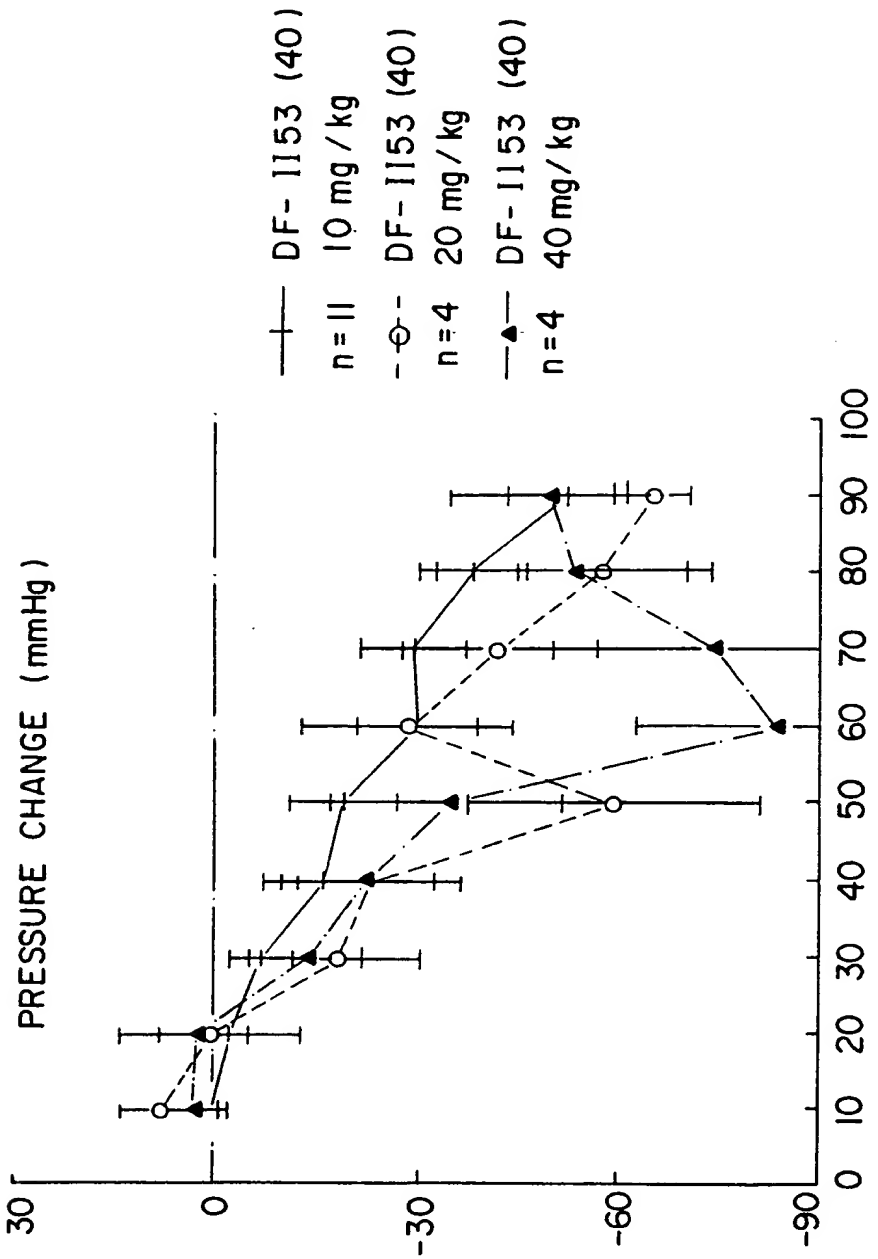
DF-1153 (40)
10 mg/kg
n=11
DF-1153 (40)
20 mg/kg
n=4
DF-1153 (40)
40 mg/kg
n=4
PRESSURE CHANGE (mmHg)
30
0
-30
-60
-90
TIME AFTER FEEDING (mins)
0
10
20
30
40
50
60
70
80
90
100

Fig. 2

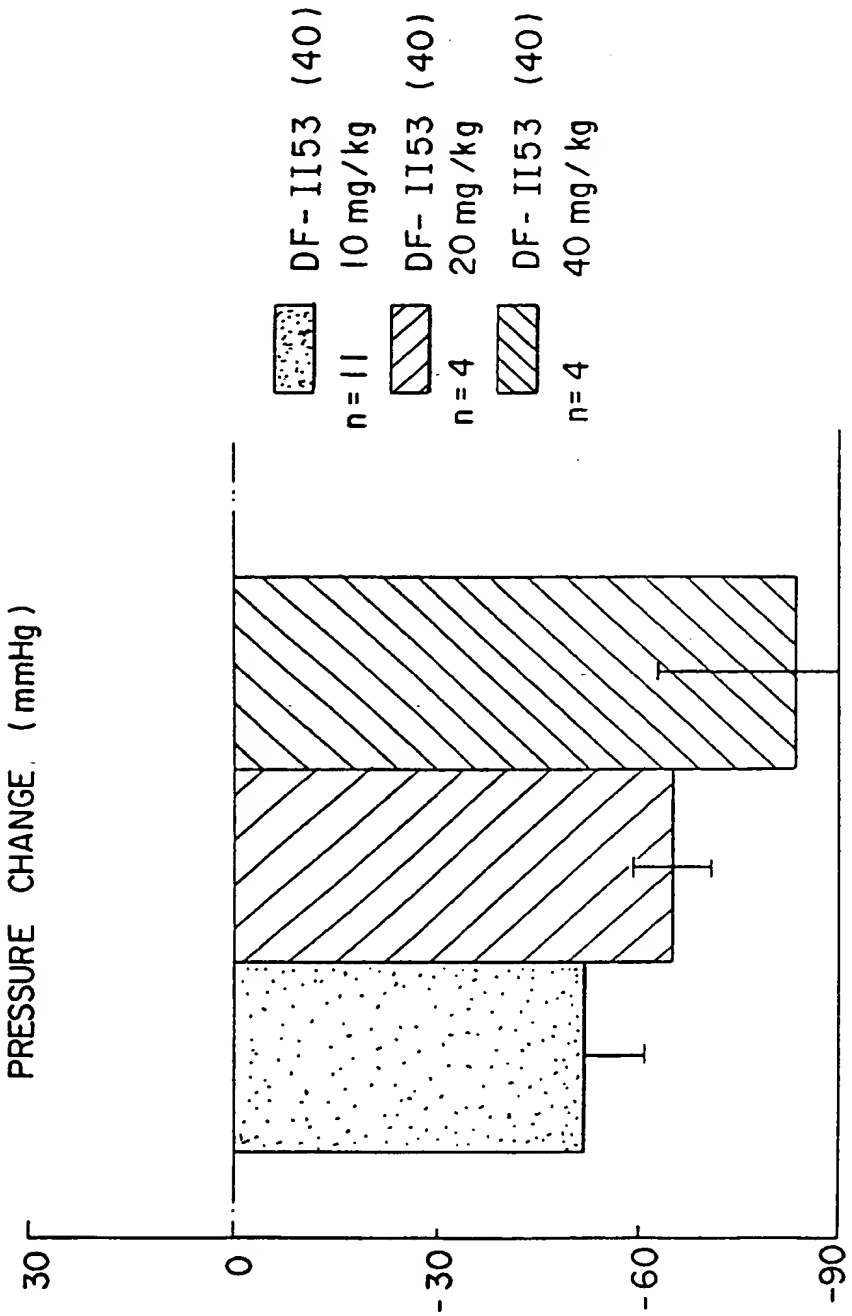
DF-1153 (40)
10 mg/kg
n=11
DF-1153 (40)
20 mg/kg
n=4
DF-1153 (40)
40 mg/kg
n=4
PRESSURE CHANGE (mmHg)
30
0
-30
-60
-90
90
90
60
TIME AFTER FEEDING. (mins)

Fig. 2a

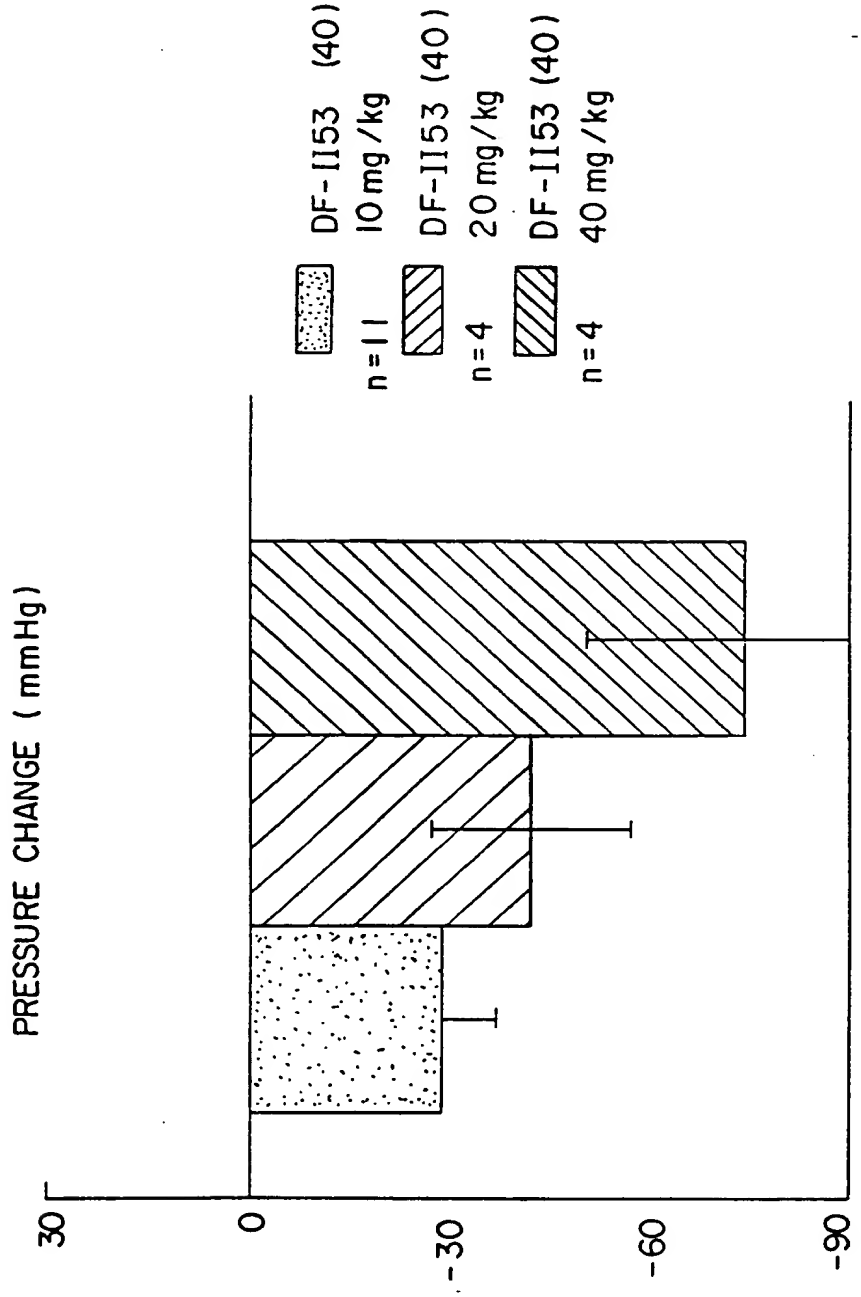
DF-1153 (40)
10 mg/kg
n=11
DF-1153 (40)
20 mg/kg
n=4
DF-1153 (40)
40 mg/kg
n=4
PRESSURE CHANGE (mmHg)
30
0
-30
-60
-90
70
TIME AFTER FEEDING (mins)

Fig. 2b

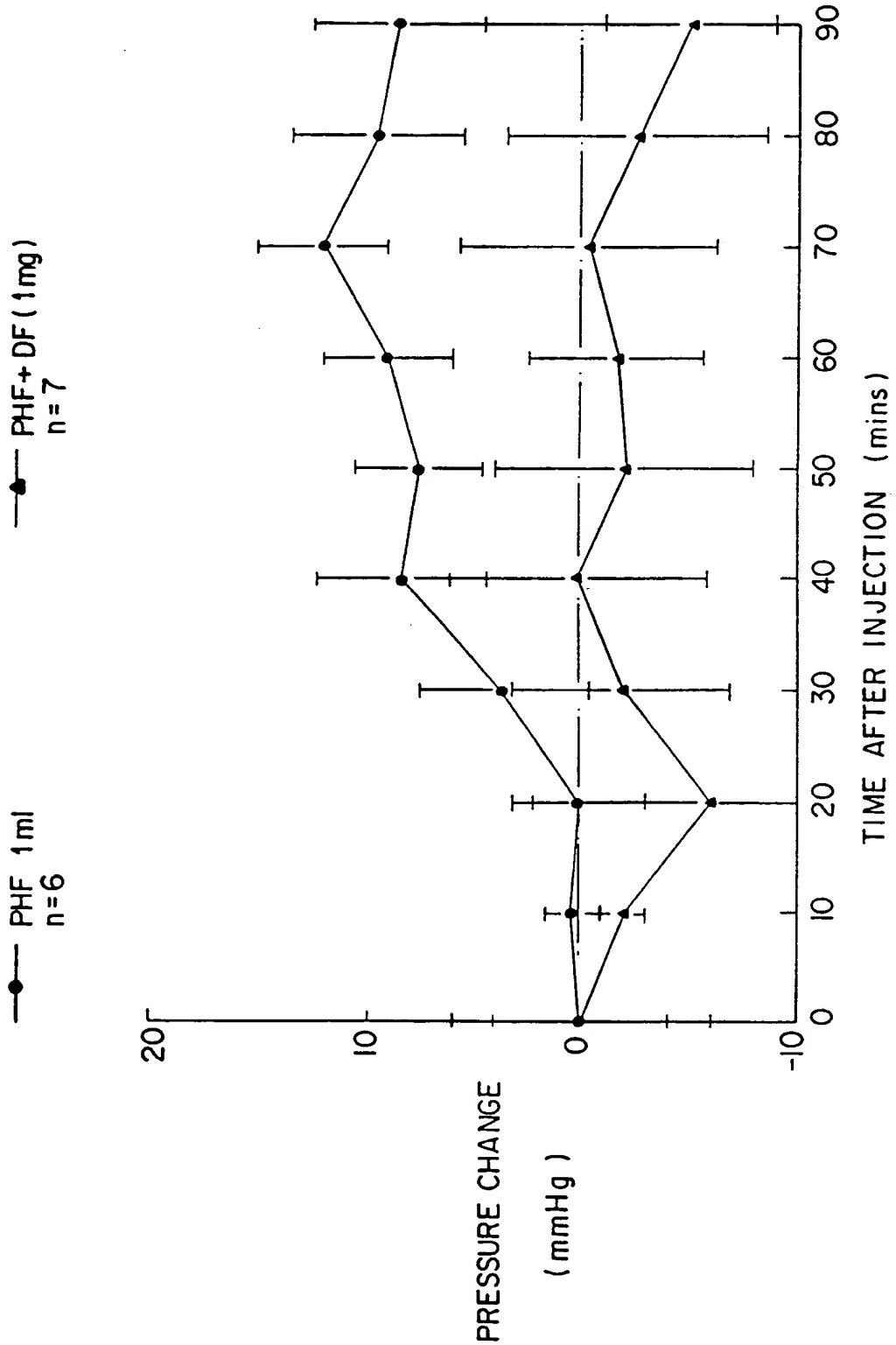
PHF+ DF (1mg)
n=7
PHF 1ml
n=6
PRESSURE CHANGE
(mmHg)
20
10
0
-10
0
10
20
30
40
50
60
70
80
90
TIME AFTER INJECTION (mins)

Fig. 3

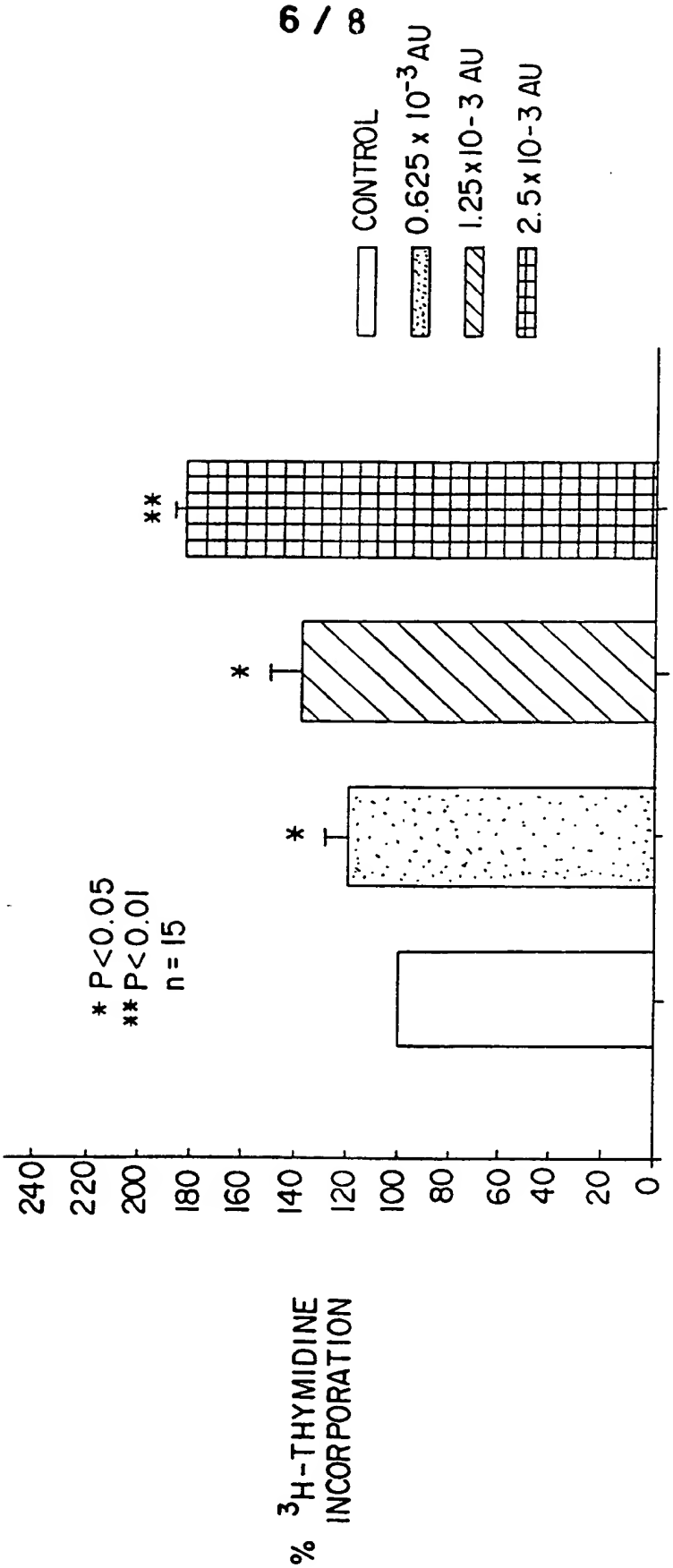
CONTROL
0.625 x 10-3 AU
1.25x10-3 AU
2.5x10-3 AU
* P<0.05
** P<0.01
n = 15
% 3H-THYMIDINE INCORPORATION
0
20
40
60
80
100
120
140
160
180
200
220
240
*
*
**

Fig. 4

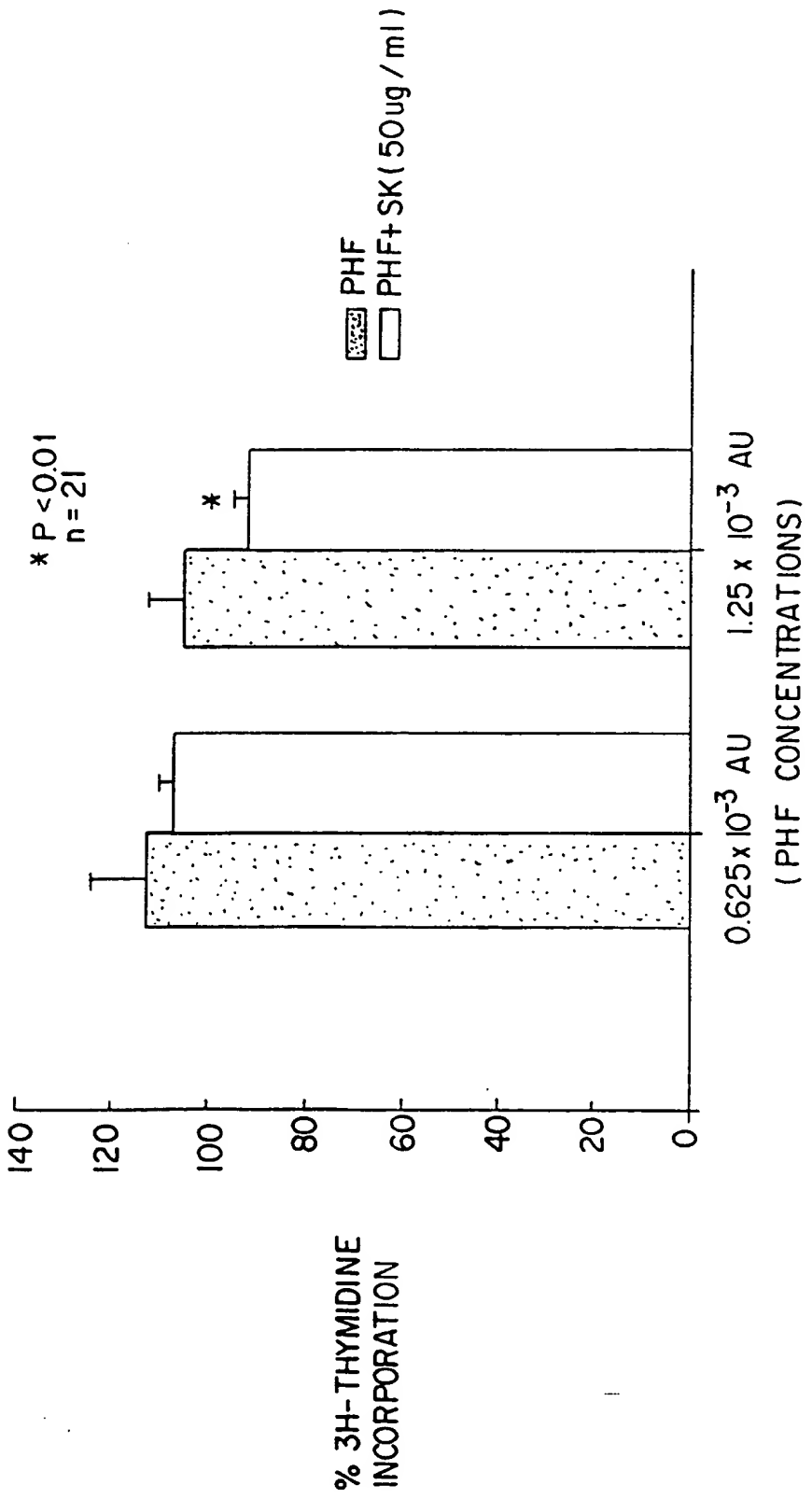
PHF
PHF+SK(50ug/ml)
* P<0.01
n=21
*
*
% 3H-THYMIDINE INCORPORATION
140
120
100
80
60
40
20
0
0.625x10-3 AU
1.25 x 10-3 AU
(PHF CONCENTRATIONS)

Fig. 5

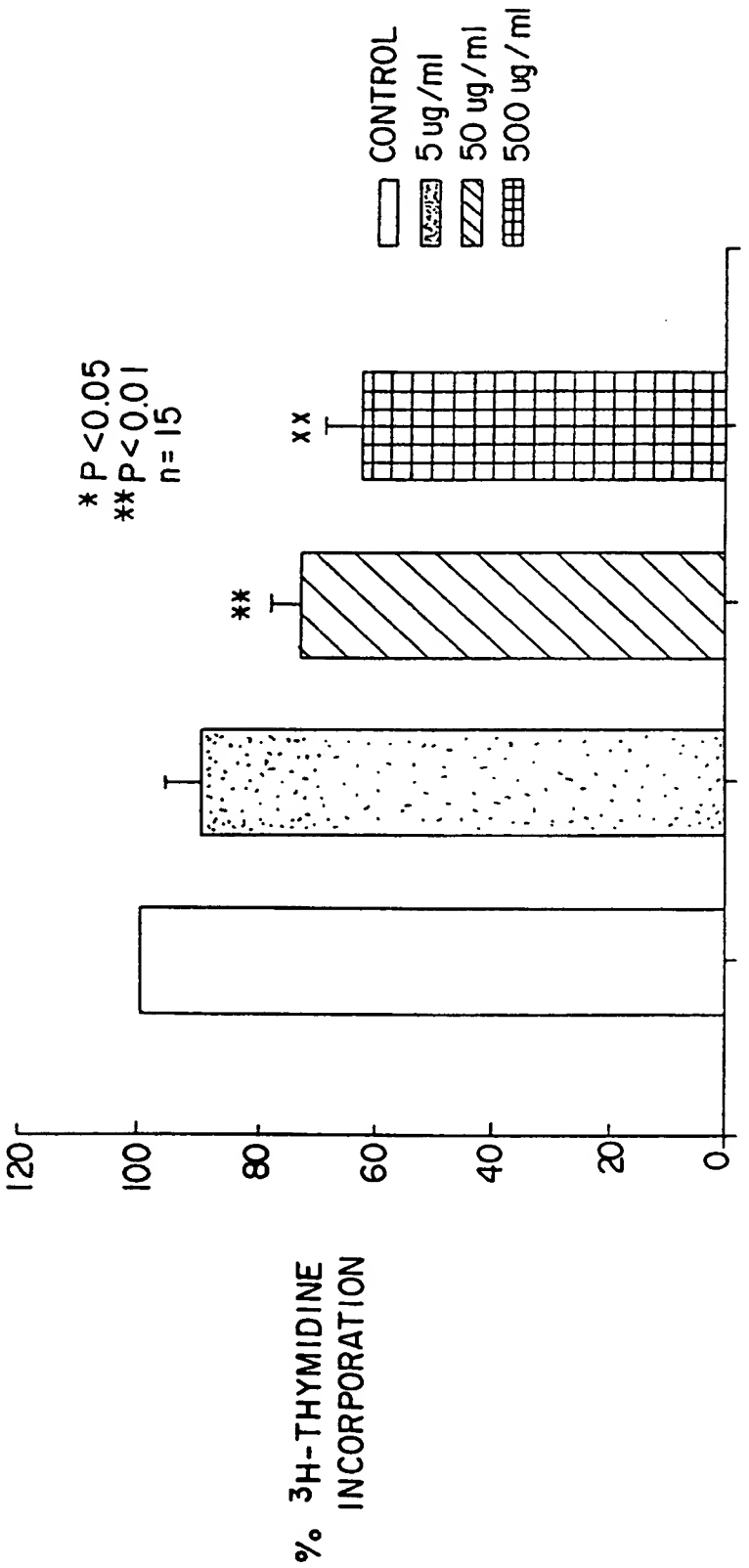
CONTROL
5 ug/ml
50 ug/ml
500 ug/ml
* P<0.05
** P<0.01
n=15
xx
**
120
100
80
60
40
20
0
% 3H-THYMIDINE INCORPORATION

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13591

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/00; A61K 35/32
US CL : 530/840, 412; 424/548

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/840, 412; 424/548

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN ON LINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,618,925 A (DUPONT et al) 08 April 1997,col. 2, lines 30-45, columns 5, 13-18.	1,2,6-8, 14,15
X	US 4,473,551 A (SCHINITSKY et al) 25 September 1984, col. 2, lines 35-55.	1,2,7,8
X	US 3,371,012 A (FURUHASHI et al) 27 February 1968, col. 3, lines 20-31.	1,2,5,7,8
A	US 4,444,752 A (PRUDDEN et al) 24 April 1984, entire document.	1-4,6-8, 14 ,15
A	US 5,075,112 A (LANE) 24 December 1991, entire document.	1-4,6-8,14, 15
A	US 5,192,664 A (PANG et al) 09 March 1993, entire document.	6,14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
21 OCTOBER 1998	03 NOV 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL BORIN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13591

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-4,6-8,14,15

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13591

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1,2,7,8,15, drawn to shark cartilage extract.
Group II, claims 3, 4, drawn to first method of use, treating hypertension.
Group III, claims 5, 9, drawn to second method of use, treating disease related to excessive PHF.
Group IV, claim 6, drawn to third method of use, treating a disease related to intracellular Ca elevation.
Group V, claim 14, drawn to fifth method of use, inhibiting smooth mucle proliferation.
Group VI, claims 10-13, drawn to method of making of product of group I.

The inventions listed as Groups I, II, VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The groups are related as product, method pof use and method of making. Group I is the technical feature that links Groups I to III. Group I is not the contribution over the prior art because it is *prima facie* obvious over the references teaching shark cartilage extract, such as, for example, taught in US Patent 5,618,925. Therefore, the lack of unity is present because the linking technical feature is not a "special technical feature" as defined by PCT Rule 13.2.

Groups III-VI are additional method of use groups.